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Effect of Nitrogen and Carbon Sources on Growth and Lipid Production from Mixotrophic Growth of *Chlorella* sp. KKU-S2

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***Abstract*—**Mixotrophic cultivation of the isolated freshwater microalgae *Chlorella* sp. KKU-S2 in batch shake flask for biomass and lipid productions, different concentration of glucose as carbon substrate, different nitrogen source and concentrations were investigated. Using 1.0g/L of $NaNO_3$ as nitrogen source, the maximum biomass yield of 10.04g/L with biomass productivity of 1.673g/L d was obtained using 40g/L glucose, while a biomass of 7.09, 8.55 and 9.45g/L with biomass productivity of 1.182, 1.425 and 1.575g/L d were found at 20, 30 and 50g/L glucose, respectively. The maximum lipid yield of 3.99g/L with lipid productivity of 0.665g/L d was obtained when 40g/L glucose was used. Lipid yield of 1.50, 3.34 and 3.66g/L with lipid productivity of 0.250, 0.557 and 0.610g/L d were found when using the initial concentration of glucose at 20, 30 and 50g/L, respectively. Process product yield ($Y_{P/S}$) of 0.078, 0.119, 0.158 and 0.094 were observed when glucose concentration was 20, 30, 40 and 50 g/L, respectively. The results obtained from the study shows that mixotrophic culture of *Chlorella* sp. KKU-S2 is a desirable cultivation process for microbial lipid and biomass production.

***Keywords*—**Mixotrophic cultivation, microalgal lipid, *Chlorella* sp. KKU-S2.

I. Introduction

MICROALGAE have the potential to generate significant quantities of biomass and oil suitable for conversion to biodiesel. Microalgae-derived biodiesel have emerged as one of the most promising alternative sources of lipid for use in biodiesel production because of their high photosynthetic efficiency to produce biomass and their higher growth rates and productivity compared to conventional crops and short generation time, use of wastewater as a source of nutrient and high oil accumulation under certain growth conditions [1], [2]. The most productive terrestrial energy crops, including palm and soybean oil, do not match the potential high productivity of microalgae [2], [3]. In addition, microalgae do not compete for land with crops used for food production, fodder and other products.

Microalgae may assume many types of metabolisms, such as photoautotrophic, heterotrophic, mixotrophic growths [3], [4]. Majority of the microalgae strains are photoautotrophic in nature and can be cultivated either in open ponds or closed system in photobioreactors using CO_2 and light as carbon and energy sources, respectively [5], this culture mode presents several disadvantages including low biomass productivity, low lipid content and long periods of cultivation. Hence, heterotrophic and mixotrophic cultures have been proposed as feasible alternatives for the production of biomass and cellular lipid accumulation [6]. Heterotrophic growth of microalgae involves the utilization of organic compounds as sole carbon and energy sources. Mixotrophic cultures of microalgae have an edge over photoautotrophic cultures as they have two energy sources as organic carbon source and light, they can simultaneously drive photoautotrophic and heterotrophic to utilize both inorganic (CO_2) and organic carbon substrates [7], [8], therefore, microalgae cultivated under mixotrophic culture synthesize compounds characteristic at high production rates of both photosynthetic and heterotrophic metabolisms of organic substrates are independent of each other [9]. Heterotrophic and mixotrophic cultures of microalgae have been reported using different carbon sources, such as glucose, sucrose, glycerol and sugarcane molasses [10], [11]. However, glucose is most commonly used for sustaining microalgae growing in the dark and was used as carbon source in mixotrophic culture of several microalgal species reaching high biomass and lipids productivity [12]-[14].

The microalgae *Chlorella* sp., showed great potentials as future industrial biodiesel producers due to their high growth rate, and their high oil contents. Carbon and nitrogen sources are the most important nutrients for mixotrophic growth and lipid production of microalgae. Therefore, in the present study, the effects of most important nutrition components, nitrogen and carbon sources, on growth and lipid productions of freshwater microalgae *Chlorella* sp. KKU-S2 was performed in batch flask cultures under mixotrophic cultivation.

II. Materials and Methods

A. Microorganisms and Culture Conditions

The microalgae *Chlorella* sp. KKU-S2 used in this study was isolated from freshwater taken from pond in the area of Khon Kaen province, northeastern Thailand [15]. The seed culture was pre-cultivated onto Bristol's medium supplemented with 20g/L glucose at 30°C in an incubator shaker at a shaking speed of 150rpm and continuous illuminated from overhead by 80W cool-white fluorescent lamps for 3 days or seed cultures were cultivated until the optical density at 680nm (OD_{680}) was 0.8. The Bristol's

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medium contained the following components (mg/L): $NaNO_3$ 250, K_2HPO_4 75, KH_2PO_4 175, $CaCl_2$ 25, NaCl 25, $MgSO_4.7H_2O$ 75, and FeCl2 0.3, $MnSO_4.2H_2O$ 0.3, $ZnSO_4$ $7H_2O$ 0.2, H_3BO_3 0.2, $CuSO_4.5H_2O$ 0.06, and pH was adjusted to 6.0 before sterilization.

B. Culture Conditions

Batch cultivations were performed in 250mL Erlenmeyer flasks, each containing 100mL of medium supplemented with glucose, flasks were inoculated with 10% (v/v) seed culture and cultivated at 30°C in rotary shaker set to 150rpm under continuous illumination by using 80W cool-white fluorescent lamps for 7 days. Periodic samples were taken from the flasks to determine the cell and lipid yields, which were then used to calculate the biomass and lipid productivities.

C. Analytical Methods

Cell growth of *Chlorella* sp. KKU-S2 was determined optical density at 680nm (OD_{680}). A standard curve was prepared by plotting dry cell weight (DCW) values (g/L) against corresponding optical density (OD_{680}) readings by using spectrophotometer. For DCW determination, microalgae preparations with known optical density were centrifuged at 5,000rpm for 5min, washed twice with sterile distilled water and dried at 90°C to a constant weight. A linear regression fit was obtained for DCW as a function of OD_{680}, y = 1.9039x + 2.2149, with R^2 value of 0.9964. There was a direct correlation between optical density and dry cell weight or dry biomass.

The culture broth was centrifuged at 5,000rpm for 5min then the supernatant was analyzed for glucose concentration according to DNS method [16]. Cellular lipids were determined by the modified method of Kwon and Rhee [17]. Biomass and Lipid productivities were calculated.

Biomass productivity (g/Ld) during the culture period was calculated from (1), where X_t was the biomass concentration (g/L) at the end of growth phase (t_t) and X_0 the initial biomass concentration (g/L) at t_0 (day):

$$Biomass\ productivity = (X_t\text{-}X_0)/\ (t_t\text{-}t_0) \quad (1)$$

Lipid productivity (g/Ld) at the end of cultivation was calculated from the (2), where P_t was the lipid yield (g/L) at the end of growth phase (t_t) and P_0 the initial lipid yield (g/L) at t_0 (day):

$$Lipid\ productivity = (P_t\text{-}P_0)/\ (t_t\text{-}t_0) \quad (2)$$

Process product yield ($Y_{P/S}$) at the end of cultivation was calculated from (3), where P was lipid yield (g/L) and S (g/L) was consumed glucose:

$$Y_{P/S} = lipid\ yield/consumed\ glucose \quad (3)$$

III. Results and Discussion

A. Effect of Different Types of Nitrogen Source on Growth and Lipid Production of Chlorella sp. KKU-S2

It has been reported that different nitrogen sources had varied influence on microbial lipid production. Therefore, effects of different nitrogen sources (inorganic nitrogen source: KNO_3, $NaNO_3$; organic nitrogen source: urea and malt extract) on cellular lipid production of *Chlorella* sp. KKU-S2 were tested. As shown in Fig. 1, among the nitrogen sources tested, malt extract supported the maximum biomass of 7.860g/L with biomass productivity of 1.310g/L d while $NaNO_3$ support the maximum lipid yield of 1.083g/L with lipid productivity of 0.155g/L d.

A biomass yield of 7.194, 7.023, 7.398g/L with biomass productivity of 1.199, 1.171, 1.233g/L d were observed when KNO_3, $NaNO_3$ and urea were used, respectively. Lipid yield of 0.517, 0.587 and 0.920g/L with lipid productivity of 0.074, 0.084 and 0.131g/L d were obtained when using KNO_3, urea and malt extract as nitrogen sources. It is worth mentioning that the organic substrate played an important role in promoting biomass accumulation of *Chlorella* sp. KKU-S2 during mixotrophic cultivation. However, with respect to the high lipid productivity, $NaNO_3$ was selected as nitrogen source for further study.

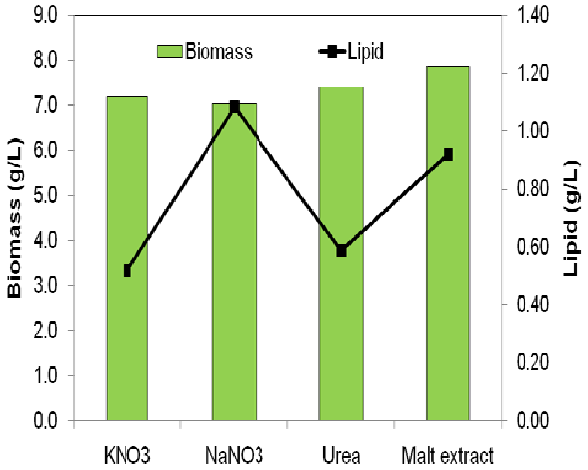

Fig. 1 Effect of different types of nitrogen sources on biomass and lipid yields from mixotrophic growth of *Chlorella* sp. KKU-S2, cultivated at 30°C for 7 days

B. Effect of Initial Nitrogen Concentration on Growth and Lipid Production of Chlorella sp. KKU-S2

According to the above results, $NaNO_3$ was selected as nitrogen source. To study of $NaNO_3$ concentration, the initial concentrations of $NaNO_3$ were 0.5g/L, 1.0g/L, 1.5g/L, and 2.0g/L, respectively. As shown in Fig. 2, higher initial nitrogen concentrations of the culture medium led to an increase in biomass concentration, the cell growth with 2.0g/L $NaNO_3$ is much better than others, the highest biomass yield of 6.941g/L with biomass productivity of 1.157g/L d, and there were no significant differences between the growths of

Chlorella sp. KKU-S2 with other three initial concentrations of $NaNO_3$. A biomass yield of 4.705, 6.314, 6.599g/L with biomass productivity of 0.784, 1.052 and 1.10g/L d were obtained when initial $NaNO_3$ was 0.5, 1.0, 1.5 and 2.0g/L, respectively.

In the experimental data, an increase in an initial concentration of $NaNO_3$ in the culture medium led to a decrease in lipid yield, the highest lipid yield of 1.099g/L with lipid productivity of 0.183g/L d was obtained when initial concentration of $NaNO_3$ was 1.0g/L. The lipid productivity decreased as $NaNO_3$ concentration increased from 1.5 to 2.0g/L, lipid yield of 0.729, 0.403g/L with lipid productivity of 0.121 and 0.067g/L d were obtained when initial $NaNO_3$ was 1.5, and 2.0g/L, respectively. Consequently, initial concentration of $NaNO_3$ at 1.0g/L, was considered to be appropriated to achieve high lipid productivity. Therefore, 1.0g/L was selected as the initial concentration of $NaNO_3$.

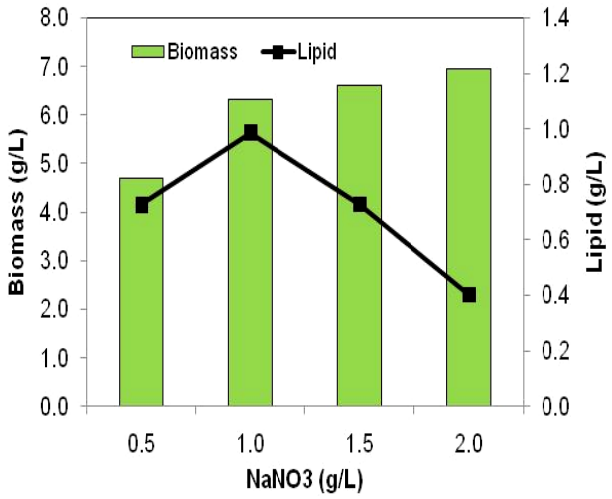

Fig. 2 Effect of $NaNO_3$ concentration on biomass and lipid yields from mixotrophic growth of *Chlorella* sp. KKU-S2, cultivated at 30°C for 7 days

C. Effect of Initial Glucose Concentration on Growth and Lipid Production of Chlorella sp. KKU-S2

Carbon and nitrogen sources refer to carbon to nitrogen molar ratio (C/N ratio) are the most important nutrients for mixotrophic culture on growth and lipid production of microalgae. The medium was Bristol with different concentrations of glucose added (20, 30, 40, 50g/L) and supplemented with 1.0g/L $NaNO_3$ as nitrogen source.

As shown in Fig. 3, both of biomass and lipid yields increased gradually with the increase of glucose from 20 to 40g/L and decreased of biomass at 50g/L glucose, and the maximum biomass yield of 10.04g/L with biomass productivity of 1.673g/L d was obtained using 40g/L glucose, while a biomass of 7.09, 8.55 and 9.45g/L with biomass productivity of 1.182, 1.425 and 1.575g/L d were found at 20, 30 and 50g/L glucose respectively.

The maximum lipid yield of 3.99g/L with lipid productivity of 0.665g/L d was obtained when 40g/L glucose was used. Lipid yield of 1.50, 3.34 and 3.66g/L with lipid productivity of 0.250, 0.557 and 0.610g/L d were found when using the initial concentration of glucose at 20, 30 and 50g/L, respectively. Further increase in glucose concentration beyond 50 resulted in a slight drop in lipid content and biomass, suggesting that a considerable glucose inhibitory effect had occurred.

Liang et al. [18] reported that, *Chlorella vulgaris*, under mixotrophic conditions, showed improved biomass production in 1% and 2% glucose while 5% and 10% were inhibitory. Indeed, C/N ratio has been found to be the major impact factor for lipid accumulation by the oleaginous microorganisms. When oleaginous microorganisms are grown with an excess of carbon and limited quantity of nitrogen, they may accumulate high concentration of cellular lipid. Cultivation of oleaginous microalgae, when nitrogen is low in the medium, the activity of nicotinamide adenine dinucleotide isocitrate dehydrogenase (NAD-IDH) decreases or even disappears from the mitochondria of the oleaginous microalgae, then tricarboxylic acid cycle is repressed, metabolism pathway altered, and protein synthesis stopped and lipid accumulation activated [19].

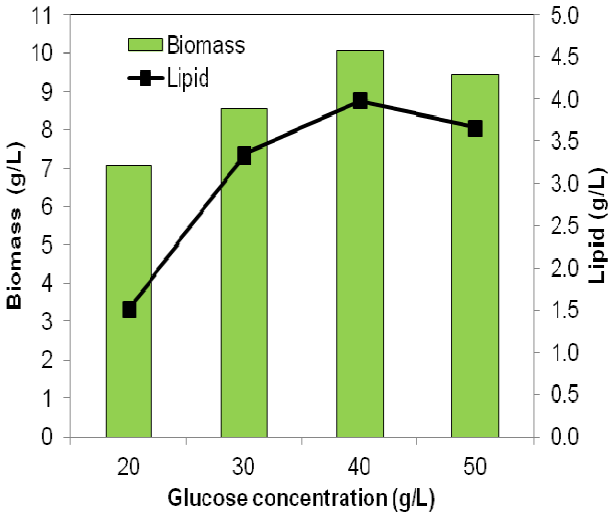

Fig. 3 Effect of initial concentrations of glucose on biomass and lipid yields from mixotrophic growth of *Chlorella* sp. KKU-S2, cultivated at 30°C for 7 days

Process product yield ($Y_{P/S}$) of 0.078, 0.119, 0.158 and 0.094 were observed when glucose concentration was 20, 30, 40 and 50g/L, respectively, the comparison of process product yield ($Y_{P/S}$) in batch cultivation at high substrate concentration, it was obvious that increase of glucose concentration resulting in decrease of this kinetic parameter, suggesting to difficult for up scaling of lipid production by microalgae due to high substrate consumption rate and high concentration of glucose with lower level of nitrogen source could be effect the cell growth, because nitrogen source supported the cell growth, thus, depleted of nitrogen may result to low biomass. To solve these phenomena, further fed-batch fermentation should investigated with initial nitrogen-rich medium to obtain high

biomass or high cell density at the early stage of cell growth, then high concentration of carbon source will feed onto culture medium for stimulate the cellular lipid accumulation. Fed-batch fermentation modes have been widely applied for microbial lipid production.

The results in this study are in agreement with a previous study, which reported that mixotrophic *Chlorella* sp. KKU-S2 growth in glucose yielded higher biomass, lipid content and productivity than cells grown under photoautotrophic and heterotrophic conditions [15], [20]. However, the biomass and lipid productivities of mixotrophic growth are significantly higher compared to photoautotrophic growths, but the high cost of organic carbon substrate as pure glucose could make mixotrophic microalgae cultivation economically unfeasible. To reduce production costs of microalgae cultivation, it is imperative to find cheap organic substrates that meet the nutritional requirements of mixotrophic microalgae. Cheap or low cost of carbon substrates such as sugars or organic carbon sources from agro-industrial by-products and wastewater, agricultural residues or cellulosic materials as well as sugarcane molasses offer great promise for mixotrophic cultivation of microalgae.

This study shows that native microalgae *Chlorella* sp. KKU-S2 isolated from freshwater was able to grow mixotrophically and high cell densities and lipid accumulation were found. In further works, optimizing of biomass will be studied by using statistically such as respond surface methodology and increasing of biomass and lipid yield of *Chlorella* sp. KKU-S2 will be investigated in a 10L reactor via mixotrophic cultivation using inexpensive raw materials such as sugarcane molasses, fermented-rice noodle wastewater or agricultural residue under batch and fed-batch cultivation modes and fatty acids profile of microalgae lipid will be studied, and then completed with the production of biodiesel by direct transesterification reactions.

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